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Response of ammonium oxidizers to the application of nitrogen fertilizer in an alpine meadow on the Qinghai-Tibetan Plateau

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ABSTRACT

The alpine meadows in Qinghai-Tibetan Plateau is an ecosystem sensitive to environmental changes. As part of a study on global climate change and the effects of N precipitation on soil microbiota, we fingerprinted ammonium oxidizing archaea (AOA) and bacteria (AOB) in relation to the N supplement in an alpine meadow in Qinghai-Tibetan Plateau. The results showed that nutrient content in the studied soil was significantly varied in different months (sampling period) but not influenced by the N supplement rate. Long-term nitrogen input dramatically changed the abundance and community composition of the AOB but exerted no obvious effect on the AOA community in the tested soil. Significant differences in the abundance and composition of AOA were recorded in different months. Our findings implied that 1) the soil fertility and physicochemical features of the studied alpine meadows in Qinghai-Tibetan Plateau were stable even under the long-term high N supply; 2) AOB might be the active nitrifiers responding to the high N supply, while the AOA were the abundant and stable nitrifiers despite the N supply; 3) sampling periods mainly affected the abundance and community composition of the nitrifiers in the tested alpine meadow; and 4) the AOB in the studied area are psychrotrophs. Therefore, despite the enhancement of plant growth, high N supplements in the tested alpine meadow ecosystems might cause more pollution to water and air, which in turn contributes to global climate change.

1. Introduction

Global climate change and anthropogenic disturbance through widespread nitrogen (N) deposition greatly affect the ecosystems in the earth (IPCC, 2007). Anthropogenic activities, including increased application of nitrogen fertilizer in agricultural ecosystems and increased fossil fuel combustion, are the main causes of increased global atmospheric nitrogen deposition (Aichner et al., 2010; Guo et al., 2010). As an essential element and one of the most important nutrients for the living things, nitrogen may constrain the growth of plants and microbial communities in terrestrial ecosystems (Howarth et al., 1997). Increased nitrogen availability greatly influences the ecosystems by alternating plant growth and biota composition (Matson et al., 2002; Zhu et al., 2015). Furthermore, changes in N availability possibly affect the soil microbial community, which also regulates many fundamental processes of N transformation in terrestrial ecosystems (Veresoglou et al., 2012).

Nitrification is a process of N transformation, which converts ammonium into nitrate via two steps: (i) oxidation of ammonia into nitrite and (ii) oxidation of nitrite into nitrate. This process enhances the leaching of NO_3^- that causes water contamination and produces compounds for denitrification, which release gases (NO, NO_2, N_2O) that exacerbate greenhouse effects. Oxidation of ammonia by ammonia oxidizing archaea (AOA) or bacteria (AOB) is the rate-limiting step in nitrification (Levy-Booth et al., 2014). AOB has long been considered the main contributor to soil ammonia oxidation. However, the discovery of AOA challenged this traditional concept (Könneke et al., 2005; Zhou et al., 2014), since great AOA abundances have been found in different terrestrial environments (Chen et al., 2014; Ouyang et al., 2016). AOA are speculated to be the important players in nitrogen cycle in low-nutrient, low-pH, and sulfide-containing environments (Erguder et al., 2009). By contrast, AOB dominate ammonia oxidation in microcosms supplied with high levels of N fertilizers (Tian et al., 2014; Xia et al., 2011). Studies have shown the changes in abundance and

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community structure of AOA and AOB in response to various environmental factors, including N fertilization regimes (Chen et al., 2008; Stempfhuber et al., 2015). Long-term nitrogen input dramatically changes the AOB community but exerts no obvious effects on the AOA community in grassland soil in Inner Mongolia (Shen et al., 2011). Moreover, the AOA community is more sensitive than AOB to different fertilization treatments in an acid red paddy soil (Chen et al., 2014). All of these findings indicate that nitrogen inputs greatly impact the community structure, abundance, and activity of AOA and AOB in soils, although the effects of N are varied in different soil systems.

As the largest geomorphological unit in the Eurasian continent, Tibetan Plateau is one of the largest alpine grasslands in the world and presents the largest and most diverse alpine grassland ecosystem in China (He et al., 2006). The abundant sunlight and precipitation in the growing season (June to September) and very low temperature in the long winter suggests that soil metabolic activities mainly occur in the growing season in this unique ecosystem. Alpine meadow soils are noted for their large reserve of N in the soils, more than 95% of which are organic forms and the remaining inorganic forms, such as nitrite, nitrate and ammonia (Zhou, 2001); N deposition enhances the biomass accumulation and CO_2 effluent in this ecosystem by increasing available N content and promoting plant growth (Fang et al., 2012; Zhu et al., 2011). In relation to the extreme environmental conditions, it could be estimated that the AOA and AOB in the soil of Tibetan alpine meadow may have community structure and biophysical features adapting to the region. However, little information is available about the soil ammonia oxidizer in this unique region (Tian et al., 2014). Focusing on the effects of N precipitation on the soil microbiota, we performed this study to evaluate the responses of AOA and AOB to N supplementation in an alpine meadow in the Tibetan Plateau, aiming at clarifying the relationships between the soil properties and abundance/community composition of ammonia-oxidizing microorganisms. The results in this study generated important implications for predicting the responses of alpine meadow ecosystems to global climate change.

2. Materials and methods

2.1. Study site and sampling

The study was conducted in the Hongyuan Alpine Meadow Ecosystem Research Station of the Chinese Academy of Sciences (32°48′N, 102°33′E, 3500 m asl) located approximately 40 km to the north of Hongyuan County on the south-eastern Tibetan Plateau. During the past 30 years, mean annual temperature in this area was 0.9 °C, with mean monthly temperatures ranging from -10.3 °C in January to 10.9 °C in July. Annual precipitation was approximately 690 mm, fluctuating greatly year by year, of which 80% occurred during the growing season, i.e., from May to August. This area has alpine meadow soil (Chinese classification), which corresponds to Cryumbrept in the US Soil Taxonomy (Shi et al., 2014). The studied area was an artificial grassland of *Elymus nutans* that had been fenced since 2003 for investigation purposes. With a vegetation cover greater than 90%, this area was dominated by *E. nutans, Koeleria macrantha*, *Vicia unijuga*, *Delphinium caeruleum* and *Allium atrosanguineum*. These five herbaceous species presented more than 80% of the total aboveground plant biomass of the meadow.

Starting in 2010, the treatments were performed in 5 × 5 m plots in triplicates in a randomized block design, and 10 m wide buffer zones around the net plots were applied. NH_4NO_3 as nitrogen fertilizer was added every year in the middle of May at the rates of 0, 2.5, 5, 10, 20 g N m^{-2} $year^{-1}$ (= 0, 25, 50, 100 and 200 kg N ha^{-1} yr^{-1}), corresponding to the treatments N0, N2.5, N5, N10, and N20, respectively. For each treatment, NH_4NO_3 was weighed, dissolved in 1 L water, and applied to each plot using a sprayer. Two passes were made across each plot to ensure an even distribution of fertilizer. The control plot received 1 L of water without nitrogen. These N doses were chosen to offer a gradient from a low to high level of N deposit based on previous related studies (Fang et al., 2012; Jiang et al., 2010; Rooney et al., 2010; Tian et al., 2014). During the experimental period, the grassland was maintained without mowing the vegetation. Soil samples were collected in triplicate from each plot by using a soil corer of 0–20 cm in depth at 2:00 pm on 10th of each month from June through September in 2013. The soil temperature was measured *in situ* for each sample. The upper layer soil (0–20 cm) was sampled based upon the fact that this is the most important zone for nutrient and microbial activity, since more than 50% of the grass root biomass are found in this zone (Schulze et al., 1996). A total of 60 samples (5 treatments × 4 months × 3 replicate) were collected. Each of the soil samples was sieved (< 2 mm) and was divided into two aliquots in sealed tubes: one aliquot was stored at −20 °C for the subsequent DNA extraction and the other aliquot was stored at 4 °C for approximately 1 week before it was used for analyses of physicochemical properties.

2.2. Soil characterization

The pH value (soil/water ratio 1:2.5), total N content (Kjeldahl, 1883), and content of soil organic matter (SOC) via dichromate oxidation (Walkley and Black, 1934) were analyzed for the soil samples. The NO_3^--N and NH_4^+-N were extracted by 1 mol L^{-1} KCl from the soil samples and were measured with continuous flow analysis by using a Lachat autoanalyzer (Lachat Instruments, Loveland, CO, USA). The humidity of soil samples was determined by the gravimetric method.

2.3. DNA extraction

Soil DNA was extracted by using the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the protocols of the manufacturer. A total of 60 extracted metagenomic DNA samples were stored at -20 °C until further processing.

2.4. Quantitative PCR assay of amoA genes

Quantitative PCR (qPCR) was performed on an Applied Biosystems (Applied Biosystems, NJ, USA) ABI 7300 sequence detection system using SYBR green detection for archaeal and bacterial *amoA* genes. The archaeal *amoA* gene amplification was carried out with primers Arch-amoAF/AR and the PCR protocol described by Francis et al. (2005), in 25 μL reaction mixtures containing 12.5 μl of 2 × SYBR Green PCR master mix (Invitrogen, NJ, USA), 5 μM of each primer and 2 μl of 5 × diluted DNA extract. The cycling parameters used were 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles of 95 °C for 30 s, 53 °C for 30 s and 72 °C for 60 s, with a detection step at the end of each cycle. et al..Bacterial *amoA* gene quantification was carried out using the PCR protocol described by (Cavagnaro et al., 2007) with primers A189 (Holmes et al., 1995) and amoA-2R' (Okano et al., 2004). Blanks (negative controls) were always run with water instead of soil DNA extract, while the positive control was the plasmid cloned the *amoA* of AOA or AOB that was used for generating the standard curves for quantifying gene copy numbers using the procedures reported by Okano et al. (2004). A melting curve analysis was performed after each assay to ensure that only the products of the desired melting temperature were generated from qPCR. The R^2 values for the standard curves were 0.993 or greater for all runs. qPCR efficiency range were 86–95% for AOA and 89–103% for AOB. All reactions were run in triplicate with a standard curve spanning 10^0–10^6 copy numbers for archaeal and bacterial *amoA* genes. The population sizes of AOA and AOB were estimated as the normalized copies per gram of dry soil.

2.5. Pyrosequencing of PCR amplified amoA genes

The archaeal and bacterial *amoA* gene fragments were amplified with the primers Arch-amoAF/Arch-amoAR (Francis et al., 2005) and

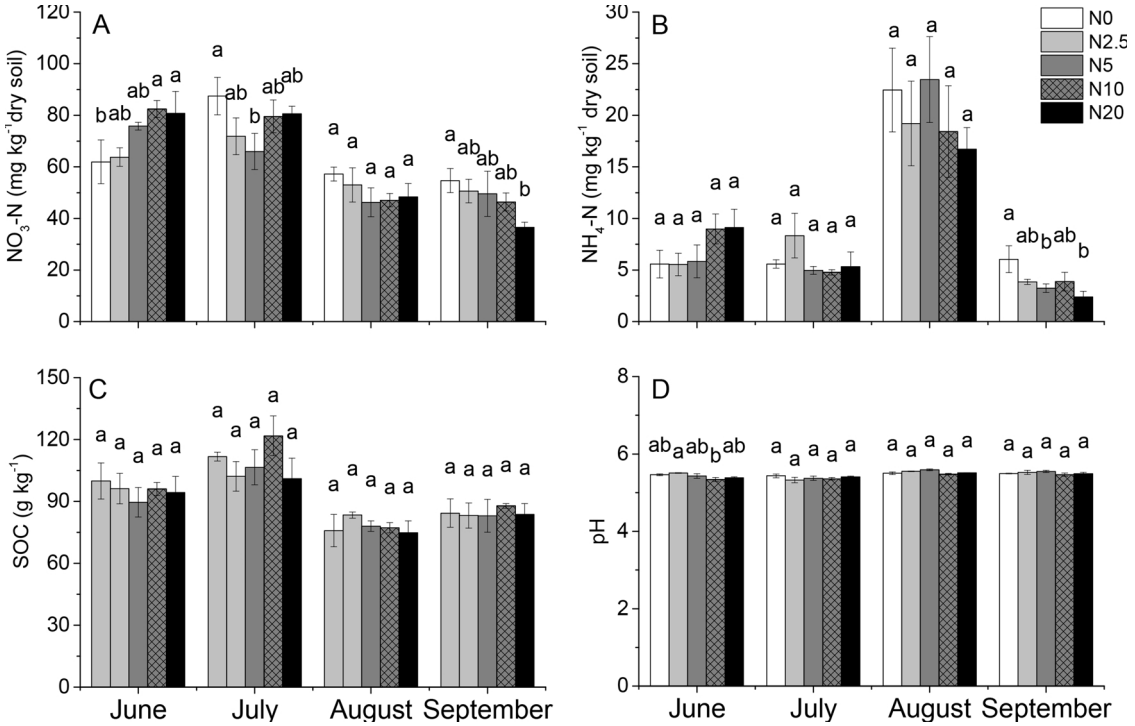

Fig. 1. Comparison of the effects of nitrogen deposition (A: nitrate-N, B: ammonium-N), soil organic carbon (C) and pH (D) in alpine meadow soil samples collected in June, July, August and September. Bars marked with distinct letters present significant difference (P = 0.05).

amoA-1F/amoA-2R (Rottauwe et al., 1997), respectively. PCR conditions were as follows: 95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min. PCR reactions were performed in a 20 μL mixture containing 4 μL of 5 × FastPfu Buffer (TransGen Biotech Co., Ltd., Beijing, China), 2 μL of 2.5 mM dNTPs (Takara), 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase (2.5 U/ml, TransGen), and 10 ng of template DNA.

After purification using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) and quantification using QuantiFluor™ -ST (Promega, U.S.), a mixture of amplicons for each sample was used for pyrosequencing on a Roche 454 GS FLX+ Titanium platform (Roche 454 Life Sciences, Branford, CT, U.S.) according to standard protocols (see Supplementary Methods) at Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP066460).

In total, 116,537 and 138,188 valid archaeal and bacterial *amoA* gene sequences were obtained from all the 60 samples, with an average of 5827 and 6909 sequences per treatment/month, respectively. The resulting sequences were processed using QIIME (version 1.17) to remove the sequences with low quality, such as those with average quality score < 20 over a 50 bp sliding window and shorter than 200 bp, with homopolymers longer than six nucleotides and containing ambiguous base calls or incorrect primer sequences. The high-quality sequences were clustered into Operational Taxonomic Units (OTUs) with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) according to the previous reports (Norman and Barrett, 2016; Tao et al., 2017; Xia et al., 2011), and chimeric sequences were identified and removed using UCHIME during the clustering analysis. The taxonomic position of each archaeal and bacterial *amoA* gene sequence was estimated by Functional Gene pipeline and repository (http://fungene.cme.msu.edu).

The rarefaction curves and the coverage percentage of all samples were calculated by using Good's method (Good, 1953). Shannon index (evenness) were calculated at cutoff values of 97% similarity using the software package QIIME (Caporaso et al., 2010). Obtained sequences were compared with those deposited in the GenBank DNA database with the BLAST search, and the related sequences were retrieved. Phylogenetic analyses were performed for the representative sequences of the OTUs and the homologous sequences obtained from the GenBank database by using MEGA version 6.0, and a neighbor-joining tree was constructed by using Kimura 2-parameter distance with 1000 bootstraps (Tamura et al., 2013). Hierarchical clustering figures were generated using custom Perl scripts.

2.6. Statistical analysis

The descriptive statistical parameters were calculated with SPSS19.0 package (IBM SPSS, Armonk). Analysis of variance (one-way ANOVA) using Duncan's Multiple Range Test (DMRT) was performed for the data of microbial abundance and soil chemical properties in different treatments. Two-way ANOVA was performed to analyze the effects of N rate, sampling periods (months), and their interaction on the microbial abundance and soil chemical properties. Correlations between nitrogen treatments, soil physicochemical properties and abundance of ammonia-oxidizers were examined by using linear regressions with a Pearson correction for multiple comparisons. Canonical correspondence analysis was conducted using CANOCO 4.5.

3. Results

3.1. Physicochemical properties of soil

In general, the soil temperature was low during the whole growth season that varied between the mean values of 9.01 °C in June and 12.54 °C in August; the soil humidity was between the mean values of 21.2% in September and 29.0% in July. The field moisture capacity of soil was approximately 110%. As shown in Fig. 1 (also Supplementary Table S1), the continuous supplement of N fertilizer in the last 4 years did not change the soil pH and SOC content during the whole growing season; but variations in the soil NO_3^--N/NH_4^+-N concentrations were observed among the treatments in some months. The soil NO_3^--N

Table 1
Summary of two-way ANOVA results (*F* values) indicating the effects of the rate of nitrogen deposition and sampling period on the soil microbial abundances and soil basic properties.

Treatments	df	Abundance of AOA	Abundance of AOB	Ratio of AOA/AOB	NO_3-N	NH_4-N	SOC	pH
N rate	4	2.062	27.479**	6.046**	0.796	0.259	0.801	2.681*
Sampling period	3	39.271**	46.075**	8.975**	34.861**	56.612**	21.163**	17.191**
N rate × Sampling period	12	0.377	2.835**	0.815	2.042*	0.978	0.521	1.327

*$p < 0.05$ and **$p < 0.01$; no asterisk denotes no significant effect was found.

content was significantly increased ($P < 0.001$) in the beginning (June) in N10 and N20 treatments, while it was significantly decreased by the end (September) in the N20 treatment. In general, the concentrations of NO_3^--N and SOC were maintained at a high level in the first two months (June and July) of sampling, and the SOC reached the peak in July; then their contents decreased dramatically in August and maintained at a low level in September in all the treatments. For all the treatments, NH_4^+-N contents maintained at low level (5-9 mg kg^{-1} dry soil) in June, July, and September but increased significantly in August up to 17-23 mg kg^{-1} of dry soil. These results demonstrated that both the N supplement level and the sampling period affected the soil NO_3^--N/NH_4^+-N contents.

The statistical analyses in our study (Table 1) indicated that NH_4^+-N concentrations and SOC were significantly influenced by sampling period (both $P < 0.001$) but not by N supplement rate. However, NO_3^--N concentrations were significantly affected by the sampling period ($P < 0.001$), but significant interactive effect ($P = 0.045$) on NO_3^--N concentrations by N supplement rate and sampling period was observed. pH was significantly affected by N supplement rate and sampling period separately ($P = 0.045$ and $P < 0.001$, respectively), and no significant interactive effect was observed between these two parameters (Table 1).

3.2. Abundance of AOA and AOB

Copy numbers of the archaeal *amoA* did not considerably change responding to the N addition levels during the growing season, although the highest copy number of *amoA* (log 7.5 copies g^{-1} dried soil) was observed in July and then decreased in all the treatments (Fig. 2A, Supplementary Table S2). For AOB, no significant difference in *amoA* copy numbers was found among the treatments in June; however, the copy numbers of the *amoA* were significantly increased under the high N rates (N10 and N20) from July to September in comparison with those in the low N rates (N0 and N2.5) (Fig. 2B). In addition, the bacterial *amoA* gene copy numbers were 1.9–4.1-fold lower than that of AOA in all the treatments and were positively correlated with that of AOA in the same treatments (Fig. 2 and Table 2). The abundance of AOB but not of AOA was positively correlated with NH_4^+-N concentration ($r = 0.352$, $P = 0.006$) but negatively correlated with soil NO_3^--N concentrations ($r = -0.304$, $P = 0.018$). Furthermore, no significant change in the AOA/AOB ratio was observed under N addition in June, but the ratio decreased in the high N rates (N10 and N20) from July to September. This ratio was positively correlated with NO_3^--N concentrations and SOC contents ($r = 0.351$, $P < 0.01$ and $r = 0.384$, $P < 0.01$) but negatively correlated with pH values ($r = -0.294$, $P < 0.05$) (Table 2).

The statistical analyses indicated the effects of N supplement rate and sampling period on the abundance of soil AOA and AOB (Table 1), revealing that the AOA abundance was significantly influenced by sampling period but not by N supplement rate. By contrast, AOB abundance was significantly affected by the interactive effect of N supplement rate and sampling period. The AOA/AOB ratio was significantly affected by N supplement rate and sampling period, and no significant interactive effect was observed between these parameters (Table 1).

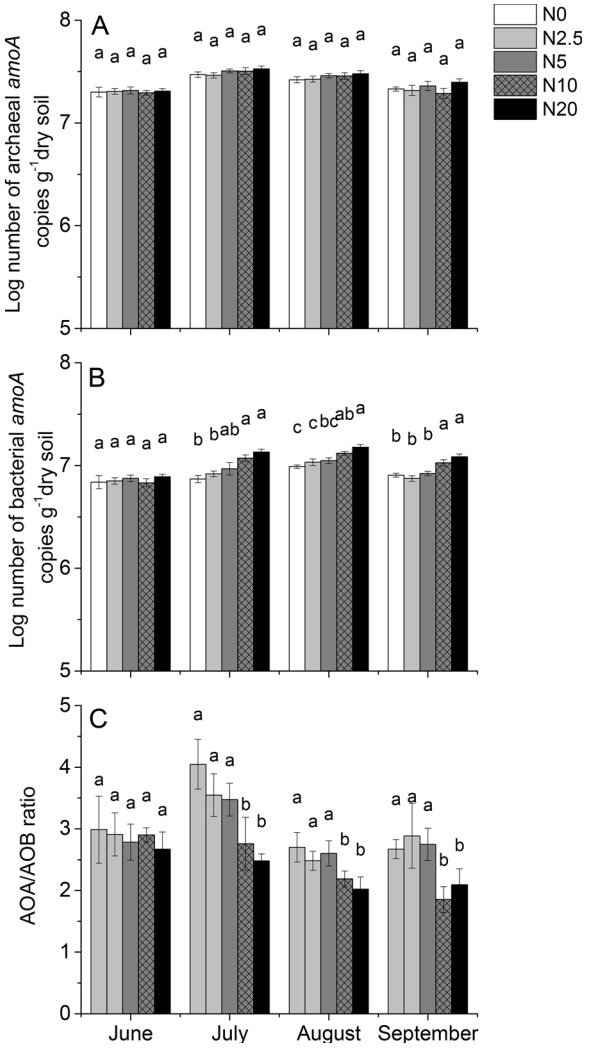

Fig. 2. Changes in AOA (A) and AOB (B) population size, and the ratio of AOA and AOB population size (C) with nitrogen deposition. Sampling points were in June, July, August and September.

3.3. Community structures of AOA and their correlation to environmental conditions

A total of 90,964 sequences were obtained after quality control with QIIME, with an average of 4548 sequences per sample and an average length of 376 bp per sequence (Supplementary Table S3). These sequence reads were clustered into 33–40 observed OTUs in each of the samples at 97% cutoff with coverage values ranging from 99.8% to 99.9% (Supplementary Table S3, also Supplementary Fig. S1). Shannon diversity indexes were higher under N0 treatment than those under N addition in June and July, but this trend was not found in August and September.

In total, 47 AOA OTUs were defined among all the samples based on the *amoA* gene sequence analysis. Most of them (46 OTUs covering 95%

Table 2
Pearson correlations of AOA and AOB abundances with soil basic properties.

	Abundance of AOA	Abundance of AOB	Ratio ofAOA/AOB	NO_3-N	NH_4-N	SOC
Abundance of AOB	0.521**					
Ratio of AOA/AOB	0.320*	-0.611**				
NO_3-N	0.007	-0.304*	0.351**			
NH_4-N	0.246	0.352**	-0.178	-0.187		
SOC	0.127	-0.223	0.384**	0.693**	-0.378**	
pH	-0.161	0.142	-0.294*	-0.646**	0.235	-0.514**

*$p < 0.05$ and **$p < 0.01$; no asterisk denotes no significant effect was found

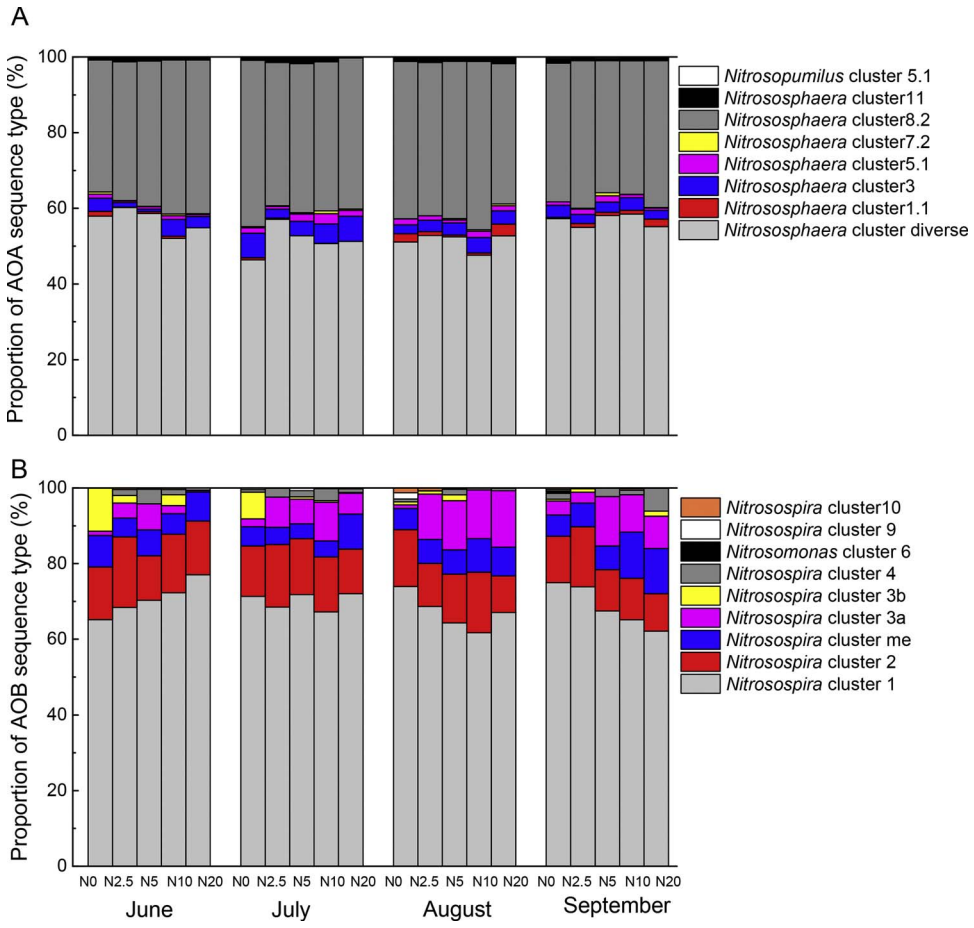

Fig. 3. Relative abundances of different lineages of AOA (A) and AOB (B) in the soils with different nitrogen deposition sampled in June, July, August and September), based on the pyrosequencing data of the *amoA* genes. Bars marked with distinct letters present significant difference (P = 0.05).

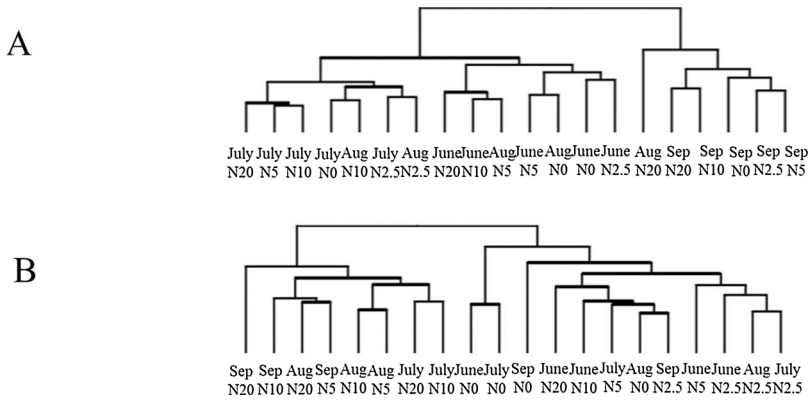

Fig. 4. Hierarchical cluster analysis based on the relative abundances of the OTUs in AOA (A) and AOB (B) data sets. The phylogenetic tree was calculated using the neighbor-joining method and the relationship among samples was determined by Bray distance and the complete clustering method.

of the 90,964 sequences) were grouped into the cluster *Nitrososphaera*, and the remaining 5% were identified as OTU2 belonging to *Nitrosopumilus* (Fig. 3A and Supplementary Fig. S2). *Nitrososphaera* subcluster 11 was only detected in September. The sequences affiliated to *Nitrososphaera* subcluster 8.2 increased under N addition in June but decreased in July. Hierarchical cluster analysis of the relative

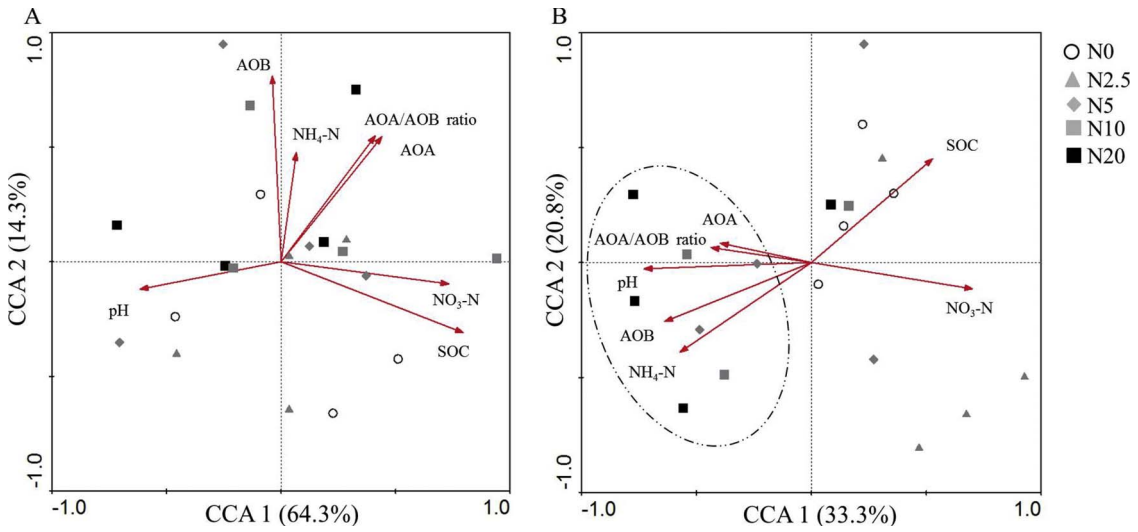

Fig. 5. CCA biplot of samples and environmental variables for AOA (A) and AOB (B) communities. Five treatments: open cycle, N0; grey triangle, N2.5; grey diamond, N5; grey square, N10; black square, N20.

abundance of the OTUs in the archaeal data sets revealed that samples from June, July and September formed three clusters according to the sampling period, while those for August were intermingled into the three clusters: the samples N0 and N5 in the cluster of June; the samples N2.5 and N10 in the cluster of July; and the sample N20 as the most divergent lineage in the cluster of September (Fig. 4A).

In canonical correspondence analysis (CCA, Fig. 5A), axis 1 describes 64.3% of the variation, and axis 2 describes 14.3% of the total AOA variation; by contrast, samples with different N supplement rates were not clearly separated in the first or second axes. The strongest determinant of the AOA community composition was SOC as indicated by the longest vector in the CCA plots, and it positively related to the AOA community, together with both the contents of NH_4^+-N and NO_3^--N. Values of pH exerted weaker effect on the AOA communities than the other environmental variables.

3.4. Community structures of AOB and their correlation to environmental conditions

A total of 138,188 valid bacterial *amoA* gene sequences were obtained in pyrosequencing and 98,994 sequences were obtained after quality control with QIIME, with an average of 4950 sequences per sample and an average length of 425 bp per sequence (Supplementary Table S4). These sequence reads were clustered into 31–48 OTUs according to the samples at 97% cutoff with coverage values ranging from 99.8%–99.9% (Supplementary Table S4).

In total, 64 bacterial OTUs were defined by the AOB *amoA* gene sequencing among all the samples. Most of them (99.9% of the sequences) were grouped into the genus *Nitrosospira,* covering 63 OTUs. The remaining 0.1% of the sequences were identified as OTU8 in cluster 6 belonging to *Nitrosomonas* (Fig. 3B and Supplementary Fig. S3). The abundance of sequences affiliated to AOB cluster 1 increased under N addition in June and decreased in August and September. Gene abundances of cluster 3a increased under N addition during June to September. Cluster 3b was the most abundant group in the N0 treatments in June and July, but it presented low content under N addition treatments from June through August. Hierarchical cluster analysis based on the relative abundance of the bacterial OTUs revealed two groups (Fig. 4B): a total of 12 samples, including all the samples in June and the samples for N0 and N2.5 treatments in each sampling period, plus the N5 sample in July, formed a cluster, while the 8 remaining samples formed the second cluster.

As shown in Fig. 5B, axis 1 describes 33.3% of the variation, and axis 2 describes 20.8% of the total AOB variation in CCA. Samples with high N rates (N10 and N20) from July to September were clearly separated on the first and second axes, similar to the result of hierarchical cluster analysis. The AOB community compositions in all the samples were clearly associated with different environmental variables. SOC was the strongest determinant related to the AOB community composition as indicated by the longest vectors in the CCA plots. The high SOC and NO_3^--N concentrations were not favorable, while higher soil pH and NH_4^+-N concentration favored the AOB communities detected in the present study.

4. Discussion

In most agroecosystems and natural grass lands, the soil N content is a limiting factor for grain and biomass production. To improving production, N-fertilizers have been widely applied in the world, which are also considered a source of contamination for soil, air and water since the exceeded N deposit can affect the microbial communities and their activities, such as nitrification and denitrification. In this study, we investigated the effects of N-supplement on the abundance and diversity of ammonium-oxidizers in a special ecosystem, the alpine meadow in eastern Tibetan Plateau. Several important findings obtained through this study are discussed here.

First, the added N was not maintained in the soil of root zone since the supply of N-fertilizer does not increase the N (NO_3^- and NH_4^+) content during the study period (Fig. 1, Supplementary Table S1), which was similar to previous observations (Tian et al., 2014). In this case, three possible outlets for the supplied N could be expected; N could be assimilated by the plants into biomass (Fang et al., 2012; Zhou, 2001; Zhu et al., 2011), released as gaseous N into the atmosphere by nitrification/denitrification procedures, and leached into subterrestrial/surface terrestrial water by precipitation (Veresoglou et al., 2012). In relation to the three outlets, ammonia-oxidizing microbes are involved in the release of gaseous N and leaching, since the final product of nitrification (NO_3^-) is the substrate for denitrification and is easy for leaching.

The fact that most of the soil N was in the form of nitrate and less in ammonia for all the samples (Fig. 1, Supplementary Table S1) might be explained 1) by the existence of efficient nitrification in the soil of tested alpine meadow, 2) by the fact that ammonium is the preferred form of N for assimilation by microbes and plants (Azam et al., 1993), and 3) by the NH_3 volatilization (Rochette et al., 2009; Craig and Wollum, 1982). The significant increase of NO_3^--N, but not NH_4^+, in

the treatments of higher doses of N (N10 and N20) in June indicated that NH_4^+ has been rapidly used by plants/microbes or transformed into NO_3^- by the nitrifiers in the early stage of N-supplement, which could reduce the NH_3 volatilization. In addition, the significant increase of NH_4^+-N (Fig. 1B) and decrease of SOC (Fig. 1C) contents in August compared with those in July imply that the decomposing microorganisms in the soil were more active in August, mineralizing the organic materials and releasing NH_4^+ to the soil.

Second, the abundance of AOA was stable compared to the N deposit levels (Fig. 2A), indicating that they were not the sensitive responders for the N-supplement in the tested soil. These results were similar to that of previously reported studies in agricultural soils (Ouyang et al., 2016) and in nitrogen-rich grassland soils (Di et al., 2009) but different from those of Tian et al. (2014) and Xu et al. (2010). Di et al. (2009) observed that, in nitrogen-rich grassland soils, neither AOA abundance nor AOA activity increased with the application of a large amount of ammonia substrate. However, Xu et al. (2010) reported that AOA abundance is reduced by increased N fertilization rates. Moreover, AOA abundance in alpine meadow soils tended to be enhanced by higher N amendment irrespective of the N forms (Tian et al., 2014). These variations in different studies may be related to the differences in soil pH and vegetation, since acid soil (pH 5.32–5.59) (Fig. 1D) and vegetation dominated by *Elymus nutans* were recorded in our study, while neutral soil (pH 7.05–7.23) and diverse flora were reported in Tian et al. (2014).

Third, AOB were the nitrifiers more actively responding to the N-supply in the tested soil (Fig. 2), although AOA were more abundant in all the samples. This finding was consistent with the previous reports, wherein AOB is frequently outnumbered by AOA in various soils (Chen et al., 2014; Ouyang et al., 2016). However, high AOA abundance does not always indicate their dominant role in nitrification (Di et al., 2009). For instance, AOB is functionally more important in microbial ammonia oxidation in an agricultural soil even though AOA was numerically more abundant than AOB (Jia and Conrad, 2009). A previous study evidenced that only AOB abundance is stimulated by addition of high amount of NH_4^+-N and positively correlated with potential nitrification rates (Tian et al., 2014). The increased AOB abundance accompanying the high N-fertilizer deposit in our study (Fig. 2) also showed that AOB are the responsive nitrifiers in the tested soil. Further analysis via CCA also illustrated that soil NH_4^+ concentrations were correlated with AOB abundance (Fig. 5). It is possible that AOB play a significant role in soils receiving inorganic N additions whereas AOA play an important role in soils with high organic N inputs (Zhou et al., 2015). Tian et al. (2014) reported that the plant growth did not affect the abundance of AOA-AOB; but our results revealed the significant effects of N deposit on AOB in July through September (Fig. 2). The reason for this difference may also be related to the soil pH and plant communities as mentioned above.

Moreover, our study showed a significant negative correlation between AOA/AOB ratios and soil pH, confirming a previous finding that the highest AOA/AOB ratios were found in sites with low pH values (Stempfhuber et al., 2015). These observations suggest that acidic soil conditions are suitable for AOA (Tourna et al., 2011). In contrast, the growth rate of AOB decreases with acid pH because at low pH values, the actual substrate (NH_3) for AOB (Chen et al., 2013; Suzuki et al., 1974) exists only at very low concentrations even if the concentration of total ammonia (NH_4^+ and NH_3) is high. Moreover, a significant positive correlation was observed between AOA:AOB ratios and SOC. AOA abundance is positively correlated with SOC in paddy soils (Li et al., 2015) and sediments (Lu et al., 2015), suggesting that AOA can assimilate organic substrates other than ammonia and thus AOA can grow mixotrophically or even heterotrophically. This view is supported by studies of *Nitrososphaera viennensis*, a soil-isolated archaeon able to grow mixotrophically on urea or ammonia with addition of pyruvate (Tourna et al., 2011).

Fourth, AOB community structures varied against the N doses and the sampling period, while AOA show obvious changes in community structure against the sampling periods (Figs. 3 and 4). Previous studies have shown that the community structures of AOA and AOB in soils are shaped by multiple environmental conditions, including soil type (Chen et al., 2010), vegetation type (Mao et al., 2011), pH (Stempfhuber et al., 2015), and nutrient level (Jiang et al., 2014a). However, little is known about the relative contributions of these environmental conditions in the overall responses of AOA and AOB community to nitrogen supplement in the alpine meadow in Qinghai-Tibet Plateau.

In this study, almost all AOA *amoA* OTUs were affiliated with *Nitrososphaera*, which is commonly detected in agricultural soils (Ouyang et al., 2016). Most sequences were affiliated with the *Nitrososphaera* cluster 8.1, a group specific for an intermediate total nitrogen and organic carbon content (Pester et al., 2012). We detected a high proportion of OTUs affiliated with *Nitrososphaera* cluster 3 in this study, which is frequently detected in acidic soils (Jiang et al., 2014b). However, *Nitrososphaera* does not always exhibit autotrophic activity in acidic soils because all the cultivated AOA within the *Nitrososphaera* lineage are neutrophilic, and the nitrification activity of these AOA is significantly reduced or even absent at pH less than 5.5 (Kim et al., 2012; Tourna et al., 2011). The *Nitrosopumilus* cluster *amoA* was absent or extremely rare (less than 0.1%) in all soils, coinciding with the low abundance or absence of the *Nitrosopumilus amoA* cluster in soils reported in other studies (Pester et al., 2012; Wessén et al., 2011). In the present study, the AOB communities were mainly affiliated with the *Nitrosospira* cluster 1, consistent with the findings in various soils (Chen et al., 2015) and sediments (Lagostina et al., 2015). It has been believed that *Nitrosospira* cluster 1 is an AOB with the highest ammonia affinities (Laanbroek et al., 2012). Ouyang et al. (2016) suggested that *Nitrosospira* cluster 3 was favored by ammonium fertilization in an agricultural soil, consistent with our finding that more AOB OTUs affiliated with the *Nitrosospira* cluster 3a under high N supplement rates in August and September. The OTUs related to the *Nitrosospira* cluster 3a included *Nitrosospira multiformis* ATCC 25196 (Choi et al., 2010), a bacterium harboring multiple copies of *amo* gene clusters, and their regulatory elements indicate responsiveness to fluctuating ammonium availability.

The present studies support results to those in the previous studies on agricultural soils, which observed that AOB community composition was altered under ammonium treatment after relatively longer incubations (Chen et al., 2014; Ouyang et al., 2016; Shen et al., 2011), whereas no shift within 4–6 weeks of incubation was observed (Avrahami et al., 2002). Considering the slow growing feature of the autolitotrophic AOB and the low temperature of the studied soils (Supplementary Table S1), it is reasonable that changes in abundance and community composition of AOB were detected after one or two months. N availability is possibly directly linked to AOB community compositions (Hynes and Germida, 2012). Unlike AOB, AOA did not show obvious changes in community structure under N additions, consistent with some previous findings (Fang et al., 2015; Shen et al., 2011) but different from others in various acidic soils (He et al., 2007; Xin et al., 2011). Therefore, the community composition alternation of both AOA and AOB responding to the N-supplement may be a function of vegetation and soil characters.

Our results show significant differences in the composition of AOA that are phylogenetically distinct in different sampling periods (Fig. 4); these results are consistent with the results in other studies (Sahan and Muyzer, 2008). Research has revealed that the seasonality of AOA community was mainly attributed to changes in soil temperature and nutrient availability (e.g., dissolved organic nitrogen and carbon) in an alpine fir forest in eastern Tibetan Plateau in China (Bernhard et al., 2010). The plant growing season in Tibetan Plateau starts in June and ends in September. Based on data obtained from the Hongyuan station, the mean temperature in July is 10.9 °C, which is the highest temperature of the year (Xu et al., 2010). NH_4^+-N and NO_3^--N concentrations, pH, and SOC were significantly influenced by plant growth

suggesting that the distinct composition of AOA community in different sampling periods is attributed to soil nutrient availability. The other possibility is that exudates from plant roots affect the community compositions of AOA and AOB. Chen et al. (2008) reported that oxygen and carbon dioxide released by rice roots into the rhizosphere were the major factors determining the changes in AOA and AOB community structures in a paddy soil. The quality and quantity of root exudates possibly varied from June to September. Studies exploring the seasonal changes in archaeal *amoA* gene composition and the environmental factors controlling such changes are scarce, and most of the available studies are molecular snapshots in specific habitats. In our study, the soil moisture was consistently low, which may favor nitrification, especially the activities of AOB, since they are aerobic microbes. The variation in soil moisture did not affected the nitrifiers in this study (Figs. 1 and 2). In addition, the soil temperature never rose above 13 °C; therefore, the AOB active in the soils would be psychrophilic microbes.

5. Conclusion

Our study demonstrated that 1) the soil fertility and physicochemical features of the studied alpine meadows in Qinghai-Tibetan Plateau were stable even under the long term high N supply; 2) AOB might be the active nitrifiers responding to the high N supply, while the AOA were the abundant and stable nitrifiers despite the N supply; 3) sampling period mainly related to the growth of plants affected the abundance and community composition of the nitrifiers in the tested alpine meadow; and 4) the AOB in the studied area are psychrotrophs. Therefore, high N supplement in the tested alpine meadow ecosystems might cause more pollution to water and air, which in turn contributes to global climate change.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.apsoil.2017.11.018

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